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"Resistance" to PSC-RANTES Revisited: Two Mutations in Human Immunodeficiency Virus Type 1 HIV-1_{SF162} or Simian-Human Immunodeficiency Virus $SHIV_{SF162\text{-}p3}$ Do Not Confer Resistance▽

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Resistance of human immunodeficiency virus type 1 (HIV-1) to small-molecule CCR5 inhibitors is well demonstrated, but resistance to macromolecular CCR5 inhibitors (e.g., PSC-RANTES) that act by both CCR5 internalization and receptor blockade had not been reported until recently (3). The report of a single simian-human immunodeficiency virus $SHIV_{SF162\text{-}p3}$ variant with one V3 and one gp41 sequence change in gp160 that conferred both altered replicative fitness and resistance to PSC-RANTES was therefore surprising. We introduced the same two mutations into both the parental HIV-1_{SF162} and the macaque-adapted $SHIV_{SF162\text{-}p3}$ and found minor differences in entry fitness but no changes in sensitivity to inhibition by either PSC-RANTES or the small-molecule allosteric inhibitor TAK-779. We attribute the earlier finding to confounding fitness effects with inhibitor sensitivity.

A recent study by Dudley et al. (3) claimed to be "the first to describe the immediate selection and infection of a drug-resistant SHIV [simian-human immunodeficiency virus] variant in the face of a protective vaginal microbicide, PSC-RANTES." The article further concluded, "This rhesus CCR5-specific/PSC-RANTES resistance selection is particularly alarming given the relative homogeneity of the $SHIV_{SF162\text{-}p3}$ stock compared to the potential exposure to a heterogeneous HIV-1 [human immunodeficiency virus type 1] population in human transmission." The study described a $SHIV_{SF162\text{-}p3}$ variant with two amino acid substitutions, K315R in the V3 loop region (present as a minor component of the p3 challenge stock) and N640D in HR2 of gp41, that conferred greater replicative fitness and greater relative resistance to both the CCR5 inhibitor PSC-RANTES (to which the single macaque harboring this variant had been exposed prior to infection) and the small-molecule allosteric CCR5 inhibitor TAK-779 (1).

While the development of HIV-1 strains resistant to small-molecule CCR5 inhibitors has been observed (11, 14), this result was surprising for several reasons. First, the inhibitory mechanism of PSC-RANTES is different from those of the small-molecule allosteric inhibitors; the ability of the macromolecule to induce profound and prolonged intracellular coreceptor sequestration, together with its ability to sterically block coreceptor use, should provide additional barriers to the development of resistant viruses that retain use of CCR5 (10). Second, this interpretation is supported by the failure to generate PSC-RANTES-resistant strains in multiple long-term *in vitro* selection studies (R.N. and D.E.M., unpublished results). Finally, the development of escape mutants in an *in vivo* setting would be expected to require sustained inhibitory concentrations of the drug at sites of replication. The Dudley et al. study was based on a single-dose experiment under conditions in which even at the highest dose used, no detectable systemic exposure occurred (6).

The determination of resistance can be confounded by the fitness of a virus isolate (7), and the claim of resistance to PSC-RANTES was surprising given that infection with the parental HIV-1_{SF162} isolate with the consensus $GPGR_{315}$ sequence is highly susceptible to PSC-RANTES inhibition (D.E.M., unpublished data) (Fig. 1 and Table 1). These concerns prompted us to determine the impact of the K315R and N640D sequence variants on the entry fitness and sensitivity of both HIV-1_{SF162} and $SHIV_{SF162\text{-}p3}$ to PSC-RANTES or TAK-779 in a single-round infection assay using either human or rhesus CCR5-expressing U87.CD4 target cells. We felt that it was important to extend the experiments of Dudley et al. (3) to HIV-1 since it is inhibition of HIV-1 infection of humans that is the intended application of a microbicide containing PSC-RANTES or related recombinant molecules (4, 12). We used site-directed mutagenesis to create three variants of the wild-type SF162 *env* sequence (R315, N640): K315, N640; R315, D640 (equivalent to the "resistant" $SHIV_{SF162\text{-}p3}$ variant from macaque 584), and K315, D640. These four *env* genes were used to complement a luciferase reporter HIV-1 construct in a standard single-round infection assay that has the advantage of

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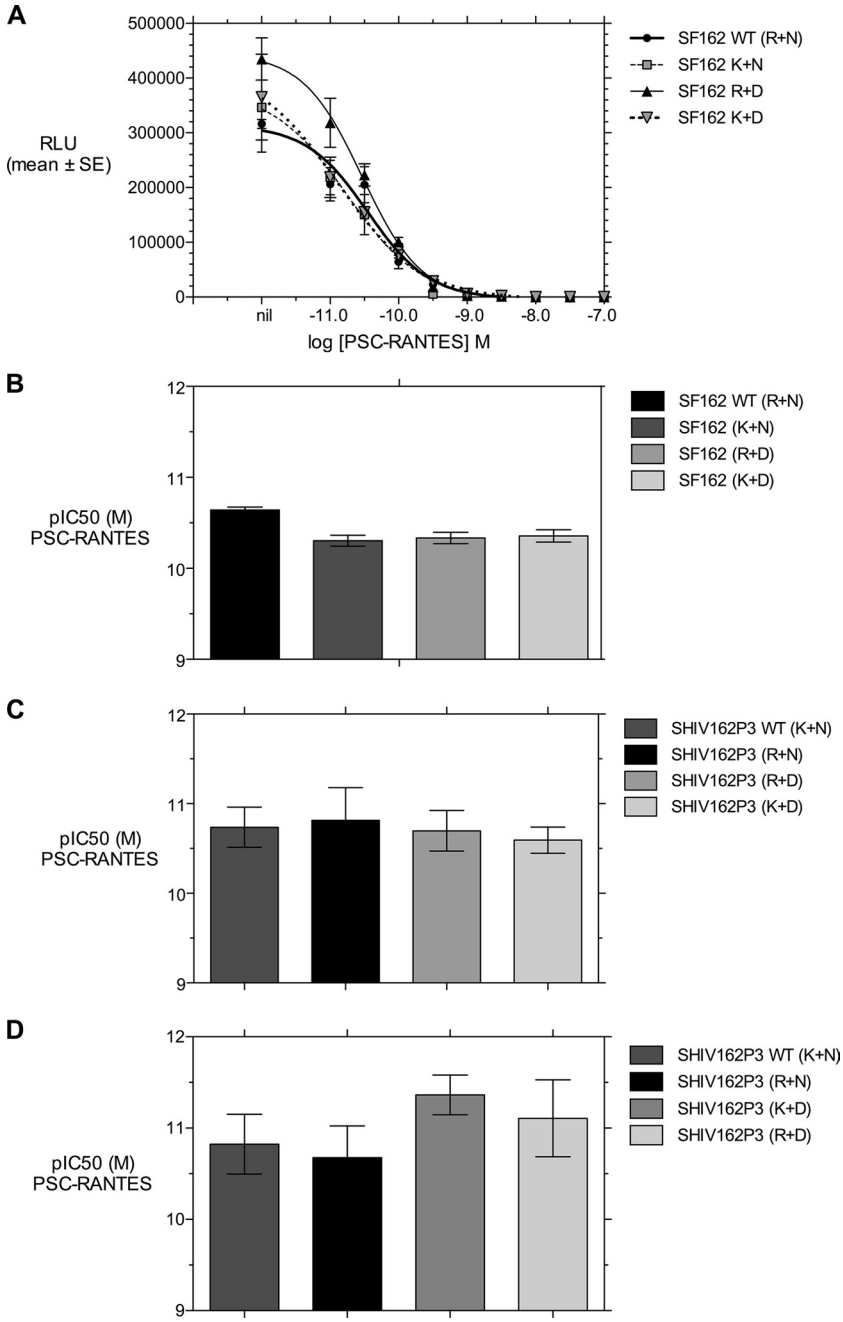

FIG. 1. Inhibition of HIV-1_{SF162} *env* mutants by PSC-RANTES. (A) Single-round infection assay performed with U87.CD4.human CCR5 target cells using the four SF162 sequence variants with half-log dilutions of PSC-RANTES added 30 min prior to infection. Data are relative light units (RLU) and are summarized in a different format in the first row of data in Table 1. (B) Means ± standard errors (SE) of the 50% inhibitory concentration of PSC-RANTES on each SF162 variant from three replicate experiments plotted as the reciprocal of the log IC_{50} (pIC_{50}) in moles. Higher pIC_{50} values indicate greater sensitivity to inhibition, but the differences depicted are not statistically significant. (C) Means ± SE of the 50% inhibitory concentrations of PSC-RANTES from three replicate experiments using the four sequence variants of $SHIV_{SF162\text{-}p3}$ with target cells expressing human CCR5. Note the different order of the columns for the $SHIV_{SF162\text{-}p3}$ variants; the "wild-type" SHIV has a different V3 sequence than the "wild-type" HIV-1_{SF162}, as well as 31 amino acid substitutions in other regions of envelope (5). (D) Means ± SE of the 50% inhibitory concentration of PSC-RANTES from three replicate experiments using the four sequence variants of $SHIV_{SF162\text{-}p3}$ with rhesus CCR5-expressing U87.CD4 target cells. WT, wild type; M, moles.

a dynamic range of up to 8 logs (9, 15). We found that D640 conferred a small but significant entry advantage over N640 in the single-cycle assay (Table 1), in agreement with the results reported by Dudley et al. (3). However, none of the SF162 mutations conferred any significant resistance to either PSC-RANTES or TAK-779 (Fig. 1 and Table 1), whether or not we corrected for the modest difference in entry efficiency. We repeated these experiments using the $SHIV_{SF162\text{-}p3}$ *env* clone

TABLE 1. HIV-1 SF162 or $SHIV_{SF162\text{-}p3}$ V3 and/or HR2 mutations do not confer resistance to CCR5 inhibitors for entry via either human or rhesus CCR5

Parameter	Result for indicated variant			
	HIV-1_{SF162} (R_{315}+N_{640}) "wild type"	HIV-1_{SF162} (K_{315}+N_{640}) "SHIVp3 like"	HIV-1_{SF162} (R_{315}+D_{640}) "resistant variant"	HIV-1_{SF162} (K_{315}+D_{640}) D_{640} gp41 change
Human CCR5				
PSC-RANTES[a] IC_{50} (pM) (95% CI[b])	36 (21–60)	18 (5.7–55)	29 (20–42)	13 (3.5–47)
r^2[c]	0.936	0.868	0.964	0.899
TAK-779 IC_{50} (nM) (95% CI)	0.24 (0.11–0.53)	0.28 (0.14–0.55)	0.39 (0.10–1.50)	0.39 (0.12–1.20)
r^2	0.896	0.919	0.858	0.842
PSC-RANTES[d] IC_{50} (pM) (95% CI)	65 (47–92)	82 (56–120)	79 (49–130)	66 (44–100)
r^2	0.937	0.893	0.918	0.937
Mean log RLU (±SEM)[e]	6.631 (0.047)	6.636 (0.019)	6.977 (0.026)	6.952 (0.023)
	$SHIV_{SF162\text{-}p3}$ (R+N) R_{315} V3 change	$SHIV_{SF162\text{-}p3}$ (K+N) SHIV "wild type"	$SHIV_{SF162\text{-}p3}$ (R+D) "resistant variant"	$SHIV_{SF162\text{-}p3}$ (K+D) D_{640} gp41 change
PSC-RANTES[a] IC_{50} (pM) (95% CI)	26 (16–43)	21 (15–28)	16 (10–28)	23 (15–35)
r^2	0.918	0.969	0.904	0.939
TAK-779 IC_{50} (nM) (95% CI)	0.69 (0.47–1.02)	0.46 (0.28–0.77)	0.60 (0.32–1.13)	0.68 (0.46–0.99)
r^2	0.949	0.921	0.877	0.949
Rhesus CCR5				
PSC-RANTES IC_{50} (pM) (95% CI)	14 (0.4–50)	65 (14.5–294)	3.09 (1.4–7.1)	9.89 (0.5–20.4)
r^2	0.690	0.615	0.839	0.866
Mean log RLU[f] (±SEM)	4.80 (0.245)	4.58 (0.519)	4.66 (0.360)	4.74 (0.517)

[a] Corrected virus input for infectivity differences; equal relative light units (RLU).
[b] The 95% confidence intervals (CI) of sigmoidal curve fitting (Prism 5.0, GraphPad) of triplicate values for each inhibitor concentration are shown.
[c] r^2, correlation coefficient for the individual data points fitted to the calculated inhibition curve.
[d] Virus input not corrected for minor increases (~0.3 log RLU) in infectivity associated with D640.
[e] RLU at equivalent p24 content of pseudoviruses containing the four variant HIV-1_{SF162} *env* sequences. Titration of input viruses showed parallel slopes of infectivity (RLU versus input p24; data not shown). SEM, standard error of the mean.
[f] Entry via rhesus CCR5 was lower than via human CCR5 because of lower expression levels following transient transfection as opposed to stable transduction; no adjustment for infectivity was necessary, because all four $SHIV_{162\text{-}p3}$ envelopes mediated similar entry.

that has the same sequence as that used in the experiments of Dudley et al. (3, 5) to determine if the finding of resistance was related to the other sequence differences between the macaque-adapted SHIV and SF162. Neither R315, D640, nor the combination of the two "resistance" mutations conferred resistance to either PSC-RANTES or TAK-779 on target cells expressing either human or rhesus CCR5 (Table 1 and Fig. 1C and D). The D640 substitution again conveyed a small entry advantage over N640 (data not shown). We thus conclude that the two mutations in $SHIV_{SF162\text{-}p3}$ that were claimed to confer resistance to PSC-RANTES using either human or rhesus CCR5 for entry were selected by replicative fitness in macaque 584 and not by drug resistance. We find no evidence that the two mutations have any impact on the PSC-RANTES sensitivity of either HIV-1_{SF162} or $SHIV_{SF162\text{-}p3}$ (Fig. 1), and we were unable to confirm the 5.5-fold increase in resistance to PSC-RANTES on target cells expressing human CCR5 or the 7-fold increase on target cells expressing rhesus CCR5 reported by Dudley et al. (3). We therefore attribute the conclusions of Dudley et al. (3) to confounding fitness effects with inhibitor sensitivity. Multiple rounds of replication in the assays employed by Dudley et al. (3) likely amplified the relatively minor differences in entry fitness that we (and they) observed and made the precise assessment of 50% inhibitory concentration (IC_{50}) values more difficult, particularly given that 10-fold dilutions of inhibitors were used in their experiments.

We observed similar inhibitory activity of PSC-RANTES on entry of both HIV-1_{SF162} and $SHIV_{SF162\text{-}p3}$ via both human and macaque CCR5, even though $SHIV_{SF162\text{-}p3}$ has many mutations in *env* that occurred during multiple passages in macaques (5), and macaque CCR5 has 7 to 8 amino acid differences from human CCR5 (8, 13), including one polymorphic site that contributes to resistance to small-molecule CCR5 inhibitors (2). While it is possible that selection for either fitness or resistance to CCR5 inhibitors may show subtle differences between SHIV-infected macaques and HIV-1 infected humans, and may even vary between macaques with different CCR5 alleles, no such differences were observed in our experiments. Although we were unable to achieve the very high levels of macaque CCR5 expression reported by Dudley et al. (3), differences in coreceptor levels would be expected to affect all four $SHIV_{SF162\text{-}p3}$ variants equally. Our results argue strongly that there is no simple mutational pathway that results in resistance to PSC-RANTES for either HIV-1_{SF162} or $SHIV_{SF162\text{-}p3}$. We conclude that the results reported by Dudley et al. (3) give no cause for concern about the development of

resistance to microbicides containing PSC-RANTES or similar compounds.

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